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GRANT NUMBER DAMD17-97-1-7251

TITLE: BCL-2, Ca, and Apoptosis in Breast Cancer

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REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

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## REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE August 1998	3. REPORT TYPE AND DATES COVERED Annual (1 Aug 97 - 31 Jul 98)	
4. TITLE AND SUBTITLE BCL-2, Ca, and Apoptosis in Breast Cancer		5. FUNDING NUMBERS DAMD17-97-1-7251	
6. AUTHOR(S) Machen, Terry E., Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California, Berkeley Berkeley, California 94720		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES		19990125 032	
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) <p>The central hypothesis of this project is that bcl-2 controls PCD and oncogenesis by altering Ca permeability by the ER/nuclear membranes, mitochondria and/or plasma membrane of mammary epithelial cells. We used fluorescence imaging and electrophysiology to measure Ca permeation and transport across the plasma membranes (and next year across ER and mitochondrial membranes) in control (and next year in bcl-2-transfected) mouse mammary epithelial cells (31EG4) before and during PCD. We perfected methods for determining the time course and morphology of living mammary cells undergoing PCD, which methods will be used later to correlate to cell and organelle Ca. We transfected 31EG4 cells with bcl-2 plasmid to begin testing the role of this gene on Ca signaling properties and PCD. Basic properties of Ca signaling due to Ca release from internal Ca stores and entry across the plasma membrane have been characterized using fluorescence imaging and patch clamp methods. These studies of bcl-2, Ca homeostasis and PCD will provide information on Ca responses to apoptotic stimuli in single mammary epithelial cells and their organelles and will also provide information about the role of bcl-2 in Ca homeostasis during PCD and in breast cancer.</p>			
14. SUBJECT TERMS Breast Cancer, bcl-2, Ca, PCD, apoptosis, Ca signaling, ER, mitochondria		15. NUMBER OF PAGES 9	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

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**ANNUAL REPORT GRANT DAMD17-97-1-7251**  
**Bcl-2, Ca and apoptosis in mammary epithelial cells**  
**Terry Machen, PI**

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**Terry Machen, PI**

**INTRODUCTION**

This proposal was designed to investigate the specific roles of and interactions between cellular Ca homeostasis and Bcl-2 in control of programmed cell death (PCD). For example, it is not known whether the changes in Ca signaling are the cause or result of PCD. In addition, there is almost no information about Ca homeostasis and its role in PCD in mammary epithelial cells. We hypothesize that Bcl-2 reduces PCD and leads to oncogenesis by altering Ca permeability by the ER/nuclear membranes, mitochondria and/or plasma membrane of mammary epithelial cells. Our goal is to use fluorescence imaging and electrophysiological methods to measure passive Ca permeation and active Ca transport across the apical and basolateral plasma membranes and also across ER and mitochondrial membranes in control and *bcl-2*-transfected mouse mammary epithelial cells (31EG4) before and during PCD. Our research to this time has involved investigating several fundamental properties of cultured mammary epithelial cells: We have perfected several methods for determining the time course and cellular morphology of mammary cells undergoing apoptosis. This has been crucial for correlation to concomitant changes of cell and organelle Ca. We have also begun transfecting 31EG4 cells with Bcl-2 plasmid to allow for testing the role of overexpression of this gene on Ca signaling properties and apoptosis of these mammary epithelial cells. Basic properties of Ca signaling due to Ca release from internal Ca stores and entry across the plasma membrane have been characterized using fluorescence imaging methods. Patch clamp methods have been used to begin characterizing Ca entry into mammary epithelial cells for correlation to the measurements of Ca entry from the use of fluorescent dyes. These studies of *bcl-2*, Ca homeostasis and PCD will provide the first investigation of asynchronous, transient and oscillatory Ca responses to apoptotic stimuli in single mammary epithelial cells and their organelles and will also provide information about the role of *bcl-2* in Ca homeostasis during PCD and in breast cancer.

**BODY**

**Technical Objective #1: Stable transfection of 31EG4 cells with *bcl-2* gene.**

Two plasmids encoding human *bcl-2* were obtained from Dr. Gabriel Nunez, University of Michigan. These plasmids, psFFV.neo-FLAG-*bcl-2* and pcDNA3-FLAG-*bcl-2*, were amplified in *E. coli*, purified and checked for the appearance of appropriate molecular weight bands by restriction enzyme digestion. The purified plasmids were transiently transfected by electroporation into HeLa cells and *bcl-2* expression was confirmed by immunofluorescent staining of the FLAG epitope that had been ligated into each plasmid adjacent to the *bcl-2* gene. The transfected HeLa cells expressed both plasmids in the expected intracellular membrane locations, but the transfection efficiency was <5% in each case.

Transfection conditions appropriate for the 31EG4 cell line were determined using a pcDNA3 plasmid containing the gene for green fluorescent protein (GFP) to allow easy determination of transfected cells. Transfection was accomplished using the liposome DNA delivery reagent, Lipofectamine Plus (GibcoBRL), according to manufacturer's instructions, and also by electroporation using the protocol for 31EG4 cells reported by Woo *et al.*, 1995. Transfection efficiencies for the GFP plasmid of 3-5% were achieved using each of these methods. Transient transfection of the *bcl-2*-containing plasmids into 31EG4 cells has so far proved unsuccessful, but we are proceeding with the establishment of stable, Bcl-2-

expressing 31EG4 cell lines using both Lipofectamine Plus and electroporation transfection methods. It is possible that the Bcl-2 expression of the transfected cells is below the detection level of our immunohistochemical assay or that we have not picked the appropriate time to assay Bcl-2 expression, so that the transient transfection results have been negative thus far. Selection of stable transfectants by antibiotic resistance should allow us to find *bcl-2* transfected cells that are expressing the protein at detectable levels. It was determined that 31EG4 cells are resistant to G418/neomycin. Selection of transfected cells is being accomplished by simultaneous transfection with a hygromycin resistance plasmid, a gift of Dr. Hsiao-Ping Moore, UC Berkeley, at 1/10 the concentration of the *bcl-2* plasmids.

Less than 1% of non-transfected 31EG4 cells cultured in the absence of the synthetic glucocorticoid, dexamethasone, express endogenous Bcl-2 that is detectable by our immunohistochemical assay. Dexamethasone treatment of 31EG4 cells promotes the formation of tight junctions, an increase in transepithelial resistance, and the expression of the milk protein casein. This differentiated phenotype may also express Bcl-2 at a higher level than that observed in cells that have not been exposed to the hormone. We will use our immunohistochemical assay to determine whether or not this is the case.

#### **Technical Objective #2: Activation and determination of time course of Programmed Cell Death (PCD) in control and *bcl-2* -transfected 31EG4 cells.**

We have found several agents that activate PCD in 31EG4 cells. Cell shrinkage, cytoplasmic blebbing, and nuclear condensation and fragmentation, all morphological indicators of PCD, were observed following treatment with the protein kinase inhibitor, staurosporine; the DNA-alkylating agent, mitomycin C; collagenase IV, which degrades the basement membrane produced by the 31EG4 cells; and thapsigargin, an inhibitor of the  $\text{Ca}^{2+}$  pump responsible for moving  $\text{Ca}^{2+}$  from the cytosol into intracellular stores. In addition to activating apoptosis, these treatments also cause breakdown of the confluent cell monolayer with mitomycin C, collagenase IV and thapsigargin treatments all producing holes in the monolayer around which cells maintain contact with each other, while staurosporine treatment caused the confluent cells to lose contact with each other but did not produce holes.

The 31EG4 mammary epithelial cells were grown to confluence on transparent, 0.2  $\mu\text{m}$  pore filters (Anopore, Nunc). Treatments were started once the cells had reached confluence and were continued for 24, 48 or 72 hours. At these time points, individual filters were removed from culture medium and placed in a phosphate-free saline buffer containing 300-500 nM Syto 11 dye and 5  $\mu\text{g}/\text{ml}$  propidium iodide. Syto 11 (Molecular Probes) is a newly-developed nucleic acid stain that fluoresces with excitation and emission wavelengths similar to those of fluorescein, that is plasma membrane permeant and that only fluoresces when bound to nucleic acid. Propidium iodide (PI) fluoresces red when bound to DNA but is plasma membrane impermeant and stains only dead cells in non-permeabilized preparations. Simultaneous use of these dyes allowed us to distinguish dead and living cells and to readily assess nuclear morphology in the Syto 11-stained, living cells. Cells were observed on a Nikon Diaphot inverted microscope using 490 nm bandpass excitation and 510 nm long pass emission filters. Propidium iodide is frequently used in permeabilized cell preparations to observe the nuclear morphology of apoptotic cells (Merlo *et al.*, 1997) The condensed, fragmented nuclei that we observed in Syto 11-stained cells were very similar to those reported in permeabilized, PI-stained cells in response to apoptosis-inducing stimuli. The fact that Syto 11 also binds RNA made it possible to see the cytoplasm in most Syto 11-stained cells. Because of this, the cytoplasmic blebbing that occurs in apoptosis was also visible in Syto 11-stained cells. To our knowledge, this is the first time that this live cell, nucleic acid stain has been used to stain and observe apoptotic cells. We will use Syto 11 to



quickly stain and ascertain whether or not particular cells whose cytoplasmic and organellar Ca responses we have monitored following the application of apoptotic stimuli have actually undergone PCD (Technical Objective #3).

Although morphological evidence of apoptosis was occasionally observed in confluent monolayers of untreated cells, staurosporine, mitomycin C, collagenase IV and thapsigargin treatments all produced significantly more apoptotic cells than were observed in control monolayers at comparable time points. For all treatments, apoptotic cells were first observed at 16-24 hours with the number increasing both with longer treatments (48-72 hours) and increased concentration of the particular treatment. Cell viability for both staurosporine and mitomycin C treatments was assessed by counting the number of cells per microscopic field over the course of a 48-hour treatment. Both staurosporine and mitomycin C caused an approximately 50% reduction in cell number over 48 hours. At no time did the number of apoptotic cells constitute the majority of cells in a field even when the number of cells per field was reduced due to monolayer breakdown. These results demonstrate that, as has previously been reported (Wolter *et al.*, 1997), PCD is asynchronous in a population of cells treated with an apoptotic stimulus. The presence in the same microscopic field of both apoptotic cells and cells not yet affected by the apoptotic stimulus will allow simultaneous assessment of intracellular Ca homeostasis in both cell types (Technical Objective #3).

The observed morphological changes following collagenase IV, staurosporine and mitomycin C treatments were confirmed to be due to apoptosis by detection of DNA strand breaks using a TUNEL (Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) assay according to manufacturer's instructions (Boehringer Mannheim), and by staining cell monolayers with fluorescently-labelled Annexin V. Annexin V is a phosphatidylserine binding protein that has been shown to label the outer leaflet of cells that have translocated this predominantly inner leaflet phospholipid to the outer leaflet of the plasma membrane during the early stages of apoptosis (Homburg *et al.*, 1995). Annexin V was purchased from Alexis Corporation and binding to 31EG4 cells was performed according to the manufacturer's instructions.

### **Technical objective #3: Measurement of Ca in cytosol ( $Ca_i$ ), ER and mitochondria during PCD in control and *bcl-2*-transfected cells.**

Fluorescence measurements of  $Ca_i$  with the Ca-sensitive dye fura 2 and digital imaging methods have shown that 31EG4 cells exhibit typical Ca signaling: there is a large intracellular pool of Ca that can be released with agonists like ATP, and once this pool of Ca is released, Ca entry across the plasma membrane into the cells is increased by at least a factor of 5. Ca entry across the plasma membrane was also activated by inhibiting the Ca ATPase of the ER with either thapsigargin or cyclopiazonic acid. These responses are characteristic of Ca release activated Ca entry channels (CRAC). As shown by changing rates of Ca entry through alteration of extracellular Ca, it appeared that CRAC was inhibited by raising  $Ca_i$ . Dose-response experiments showed CRAC was sensitive to the trivalent cation La, with a concentration dependence (50% block at 90-500 nM) similar to what has been observed by others for CRAC (Kerschbaum and Cahalan, 1998). Preliminary experiments utilizing mitochondrial inhibitors showed that mitochondria in 31EG4 cells also store Ca and may play a role in regulating CRAC, which could provide a critical link among Bcl-2, mitochondria (where Bcl-2 is localized), CRAC, Ca entry and PCD.

Since CRAC may be crucial for initiating PCD and Bcl-2 may play a role in regulating CRAC, we performed further electrophysiological experiments in an attempt to characterize this Ca entry pathway in more detail. Initial patch clamp were conducted using cell-attached patches that contained no Ca but used Na as the charge carrier, and a small (2 pS) channel that was activated by thapsigargin was identified. This channel was, however, quite difficult

to identify routinely. We therefore adopted the whole cell patch clamp method, while clamping  $Ca_i$  to low levels using either of the Ca buffers EGTA or BAPTA and utilizing Na as a charge carrier (similar to approach used by Kerschbaum and Cahalan, 1998). Small currents (10-20 pA) that were stimulated by thapsigargin and exhibited reversal potentials consistent with Na-carrying channels were activated in some cases. These experiments will be continued in control and *bcl-2* transfected cells.

## CONCLUSIONS

1. Mammary epithelial cells (31EG4) cultured under physiological conditions as a confluent monolayer on permeable supports undergo typical PCD over the time course 16-48 hrs when induced by a variety of treatments. Fluorescence methods, particularly the use of Syto 11, allow monitoring the time course and progression of PCD in living cells so that cell physiology can be compared in control and PCD-induced cells in the same microscopic field. Collagenase IV treatment of 31EG4 cells grown on permeable supports provides a model for the PCD that accompanies mammary gland involution.
2. Short term (secs) Ca regulation in 31EG4 cells is dominated by Ca release from internal stores, while long term (mins-hrs) is controlled by Ca entry (through CRAC) across the plasma membrane. Interactions among CRAC and mitochondria may provide a link among mitochondria, Bcl-2, CRAC, Ca entry and PCD.

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## PUBLICATION

Abstract to American Society of Cell Biologists to be presented December, 1998, San Francisco, CA

Apoptosis Induction in Cultured Mammary Epithelial Cells  
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Treating confluent 31EG4 mouse mammary epithelial cells with several agents led to disruption of the cell monolayer and morphological evidence of apoptosis. Cell shrinkage, cytoplasmic blebbing, and nuclear condensation and fragmentation were observed in live cells stained with the fluorescent nucleic acid stain Syto 11 following treatment with collagenase IV, staurosporine or mitomycin C. Staurosporine and mitomycin C treatment caused a substantial loss of cell viability over 48 hours. Cell death was due to apoptosis because all three treatments gave positive staining of live cells with annexin V, indicating the translocation of phosphatidylserine from the inside to the outside of the plasma membrane, an early event in apoptosis. In addition, all three treatments produced nicked nuclear DNA, also characteristic of apoptosis. Mammary gland involution and apoptosis during weaning involves increased production of the extracellular matrix-degrading enzyme stromelysin. Basement membrane degradation by collagenase IV produced holes in the confluent 31EG4 cell monolayer. These holes were ringed by stretched cells that maintained contact with neighboring cells, but that had lost contact with the subcellular matrix. Our data suggest that collagenase IV treatment of cultured 31EG4 cells provides a model for the apoptosis that accompanies mammary gland involution.